Seasonal changes of DNA fragmentation and quality of raw and cold-stored stallion spermatozoa

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Abstract
In this study annual fluctuations of DNA fragmentation and quality of cold-stored equine sperm were evaluated. Ejaculates were collected weekly during one year from 15 stallions. Ejaculate volume, sperm concentration and total sperm count were determined and semen was then extended and cold-stored for 48 h. Sperm motility was evaluated by CASA before and after 24 as well as 48 h of cold storage. In addition, the percentages of sperm with intact plasma membrane and acrosome (PMAI %) and with low intracellular Ca^{2+} level were determined in cold-stored semen (24 h, 48 h). SCSA^{TM} was performed to assess mean DFI, SD of DFI and % DFI in raw frozen-thawed as well as in extended sperm after 24 and 48 h of storage. The month of semen collection affected ($P<0.05$) all parameters evaluated in raw semen and all criteria except progressive motility as well as rapid cells in semen stored for 24 and 48 h, respectively. Ejaculate volume was higher and sperm concentration lower in summer compared to winter and motility lower in July than in any other month of the year ($P<0.05$). In semen processed in April and stored for 24 h the percentage of rapid cells was improved compared to January and after 48 h of storage progressive motility (%) was higher in January and October than in July ($P<0.05$). After 24 h of cold storage PMAI % was higher in October than in January and after 48 h values were higher in September compared to January and February as well as from April to July ($P<0.05$). Regarding sperm with low intracellular Ca^{+2} level (%) after storage for 24 and 48 h, higher values were measured in winter and in October compared to April, June and July ($P<0.01$). Seasonal changes in DNA fragmentation were most evident with respect to mean DFI. In raw frozen-thawed semen mean DFI was lower from August to November than in June and July ($P<0.001$). Values were lower during winter compared to spring and early summer ($P<0.05$) and lower in December than from April to September ($P<0.001$). After 24 h of cold storage mean DFI was lower in September and October when compared to January, February, May, July and November ($P<0.05$) and after 48 h storage mean DFI was reduced in spring and autumn compared to February, June and July ($P<0.05$). In conclusion, a seasonal effect was evident on semen characteristics of raw and cold-stored sperm. Semen quality was impaired in midsummer when low sperm motility and viability were combined with an elevated DNA fragmentation and Ca^{2+} level of sperm.

Keywords: stallion, season, sperm, semen quality, sperm chromatin

1. Introduction

The horse is a long-day breeder and considering annual variation in semen quality allows to optimize reproductive efficiency of stallions [1, 2]. Seasonal influences on endocrine testicular function [3-5] as well as on common semen characteristics of fresh and frozen-thawed semen are well documented in the literature [6-8]. Regarding cold-stored semen, however, only limited information is available comparing the quality between February and November [9] or between spring and summer [10].

With the establishment of flow cytometry in semen analysis, new fertility-related sperm parameters could be identified. The sperm chromatin structure assay (SCSA™) is one of the most widely used methods for the evaluation of sperm chromatin stability and was validated as highly useful test for determination of the fertilizing capacity of mammalian sperm [11]. Also in equine sperm it could be shown that the DNA integrity determined by SCSA™ is related to fertility [12, 13]. Regarding seasonal changes of chromatin structure in equine sperm the information in the literature is sparse. Blottner et al. [14], comparing sperm DNA fragmentation between May and December, found a slightly enhanced susceptibility of sperm DNA to denaturation in December during the non-breeding season. These findings contrast with results of a recent study using cryopreserved semen stored for 10 years where no seasonal pattern was apparent in semen collected every other week during one year [15]. Because of little and inconsistent information, the aim of this study was to investigate annual variation of DNA integrity and of the quality of raw and cold-stored stallion sperm using computer-assisted motility analysis and flow cytometry.

2. Materials and Methods

2.1 Animals and semen collection

Fifteen healthy Franches-Montagnes stallions, aged between 5 and 19 years, from the Swiss National Stud Farm of Agroscope in Avenches (Switzerland) were used for the experiment. The animals were kept in box stalls under natural climate and photoperiod, and were fed hay, oats and pellets supplemented with minerals. Water was available ad libitum. Before starting the experiment, stallions were trained to mount a dummy and extragonadal sperm reserves were minimized by daily semen collections for seven days. Thereafter, ejaculates were collected once a week (always on the same weekday, between 7:30 am and 12 am) for one year (March 2013 to February 2014). During the breeding season (March to July) 10 of the 15 stallions were also used occasionally for natural mating or artificial insemination. Animal experimentation was performed in accordance with the relevant guidelines and regulations (permit # 2667.0; Service Vétérinaire Etat de Vaud and Swiss Confederation).

2.2 Semen processing and examination

Immediately after semen collection, the volume of the gel-free ejaculate was estimated, sperm concentration determined by a Nucleocounter SP-100 (ChemoMetec A/S, Allerod, Denmark) and the semen placed in a water bath at 38 °C. Aliquots of raw semen were filled in straws (0.5 mL), frozen in nitrogen vapor during 5 min and plunged in liquid nitrogen for storage and later SCSA™ evaluation. The rest of the semen was diluted with INRA96™ (IMV Technologies, L'Aigle, France) to a concentration of $30x10^6$ spermatozoa/mL and samples placed in an Equitainer™ (Hamilton Thorne Inc., Beverly, U.S.A) for 24 h. Thereafter the semen samples were transferred to a refrigerator (4 °C) and stored for further 24 h.

2.3 Sperm motility assessment with CASA

The IVOS CASA system (Hamilton Thorne Inc., Beverly, U.S.A.) was used to assess sperm motility. Samples of extended semen ($30x10^6$ spermatozoa/mL) were incubated at 38 °C and analyzed after 10 min. For the measurements a 20 µm deep counting chamber slide (Leja, Nieuw-Vennep, the Netherlands) was loaded with 6 µL of the sample and a minimum of 1000

cells were analyzed in no less than eight randomly selected fields, with 30 frames acquired per field at a frame rate of 60 Hz using standardized settings for stallion semen [16]. Sperm with straightness ≥70% and VAP ≥50 μm/s were considered progressively motile, while sperm with VAP ≥ 50 μm/s were classified as rapid cells. CASA was performed in extended semen immediately after collection (0 h) and after 24 as well 48 h of cold storage.

2.4 Flow cytometry analyses

2.4.1 Integrity of plasma/acrosome membrane and intracellular Ca^{2+} level

Evaluation of the plasma membrane and acrosome integrity as well as of the intracellular Ca^{2+} level of spermatozoa were performed using a Cell Lab Quanta SC MPL flow cytometer, operated by the Cell Lab Quanta SC Software for instrument Control Data Acquisition (Beckman Coulter Inc., Nyon, Switzerland), which was equipped with a solid state LASER exciting at 488 nm and emission filters detecting green, orange and red fluorescence at 525, 590, 670 nm, respectively. Flow rate was set to 500 cells/s and for each sample 10000 events were analyzed. Membrane integrity and acrosomal status of spermatozoa were evaluated after double staining with propidium iodide (PI) and peanut agglutinin conjugated with fluorescein isothiocyanate (FITC-PNA) [17, 18]. Five μl of the semen previously diluted in 238.5 μL Tyrode`s solution (100 mM NaCl, 3.1 mM KCl, 2.0 mM $CaCl_2$, 0.4 mM $MgCl_2$, 0.3 mM NaH_2PO_4, 25 mM $NaHCO_3$, 21.6 mM $C_3H_5NaO_3$, 1.0 mM $C_3H_3NaO_3$, 10 mM $C_8H_{18}N_2O_4S$, 50 mg/L Gentamicin, 0.5 g/L Polyvinyl alcohol, 0.5 g/L Polyvinylpyrrolidone; pH 7.4, 320±5 mOsm; all chemicals were obtained from Sigma-Aldrich, Buchs, Switzerland) to a final concentration of 0.6 x 10^6 spermatozoa/mL were stained by adding 1.5 μL of 2,99-mM PI (Sigma-Aldrich, Buchs, Switzerland) and 5 μL FITC-PNA (100 μg/mL) (Sigma-Aldrich Buchs, Switzerland). Prepared stained samples were incubated at 38 °C for 15 min before being assessed with flow cytometry. The percentage of membrane and acrosome intact spermatozoa (PMAI %) was determined in extended semen after 24 and 48 h of cold storage.
For determination of intracellular Ca^{2+} level a combined staining with PI and Fluo-4 AM was used [19]. Eight μL of 1-mM Fluo-4 AM (Life Technologies Europe B.V., Zug, Switzerland) and 1.5 μL of 2,99-mM PI (Sigma-Aldrich, Buchs, Switzerland) were added to 5 μL of semen diluted in 234.5 μL Tyrode`s medium to a final concentration of 0.6 x 10^6 spermatozoa/mL. After incubation for 15 min at 38 °C samples were analyzed and the percentage of viable cells with low intracellular Ca^{2+} level was determined after excluding PI-positive cells in extended semen after 24 and 48 h of cold storage.

2.4.2 DNA integrity

To assess DNA integrity of spermatozoa, the sperm chromatin structure assay (SCSA™) was performed using a Coulter EPICS XL flow cytometer driven by EXPO32 ADC XL 4 Color™ software (Beckman Coulter Inc., Krefeld, Germany). Cells were excited by a 488 nm Argon LASER and the emitted green, orange or red fluorescence was captured at 525, 575 or 620 nm, respectively. A total of 10000 events were analyzed for each sample at a flow rate of 200 cells/s. Data analysis and computation of SCSA™ parameters were performed using the 4.07.0005 version of FCS EXPRESS Flow Cytometry Research Edition software (De Novo Software, Glendale, USA).
SCSA™ was performed according to Evenson and Jost [20]. Chemicals used for preparations were obtained from Sigma-Aldrich (Buchs, Switzerland). Acridine orange (AO) was purchased from Polysciences Europe GmbH (Eppelheim, Germany) and AO staining buffer was prepared (6.0 μg AO/mL, 0.1-M citric acid, 0.2-M Na_2HPO_4, 1-mM EDTA, 0.15-M NaCl, pH 6.0). Acid-induced denaturation of sperm DNA in situ was achieved by adding 400 μL of a detergent solution (0.15-M NaCl, 0.08-N HCl, 0.1% Triton-X 100 pH 1.2) to 200 μL of semen previously diluted with TNE buffer (0.15-M NaCl, 1-mM EDTA, 0.01-M tris buffer, pH 7.4) to a final concentration of 1-2x10^6 sperm/mL. Following thorough mixing of

the sample and 30-second incubation, 1.2 mL of AO staining buffer were added and stained samples were flow cytometrically assessed after exactly 3 min. A reference sample was processed in the same manner and saved as control for the procedure. The extent of DNA fragmentation of spermatozoa was expressed as DNA fragmentation index (DFI) quantified by the ratio of red single strand to the total single (red) + double (green) strand DNA fluorescence. To describe DFI distribution the mean DFI, variation (SD of DFI) and the percentage of the cells outside the main population (% DFI) were determined [11, 20, 21]. DNA fragmentation was evaluated in raw (0 h) and in extended semen after 24 and 48 h of cold storage. To simplify the protocol for raw semen, samples were frozen and stored in liquid nitrogen and SCSA^{TM} performed at the end of the study in frozen-thawed (38 °C, 30 s) sperm.

2.5 Testosterone analysis

Blood samples were collected by jugular venipuncture the day after semen collection between 11 and 12 am, centrifuged (5000 x g, 10 min) and serum frozen (-80 °C) until analysis. Serum testosterone concentrations were determined by radioimmunoassay [3]. The detection limit of the assay was < 0.1 ng/mL. Inter- and intraassay coefficients of variation were 7.8 % and 9.0 %, respectively.

2.6 Microclimatic conditions

Daily ambient temperature, relative humidity and hours of sunshine were recorded by a meteorological station in the region (Payerne, 46° 49`N, 06° 57`E) where the stallions were located.

2.7 Statistical analysis

Statistical analysis of the experimental data was performed using the R version 3.1.3 language for statistical computing [22]. Mean values and standard deviation (SD) were used to summarize the distribution of characteristics; descriptive statistics were computed in relation to the month of ejaculate collection and the time of semen storage (0, 24 and 48 h). The fixed effect of month on sperm characteristics at 0, 24 and 48 h was assessed by means of linear mixed-effects models. The effect of stallion was added as a random effect repeated over a series of weekly evaluations. The intercept of the relationship between sperm characteristics and the month of ejaculate collection was allowed to vary across stallions. Given the repeated-measurements design of the study, the covariance structure of linear models was assumed to be a first-order autoregressive structure. The -2 log-likelihood criterion (at 0.05 significance level) was used to assess the goodness of fit of models containing the fixed effect of month in comparison to a basic model including only the random effect of stallion. Model parameters were estimated using the method of least squares. Linear mixed-effects modeling was performed using the lme function of the nlme statistical package, whereas the plot and qqnorm functions were used to visually inspect the distribution of model residuals [23]. Correlation coefficients between the assessed variables were calculated using the methodology suggested by Bland and Altman for repeated observations [24].

3. Results

3.2. Effect of month of ejaculate collection on semen characteristics

The month of semen collection affected ($P<0.05$) all quantitative parameters determined in raw semen (Table 1). Ejaculates collected from March to September showed lower sperm concentrations compared to winter (January and February) ($P<0.01$). Concentrations measured from April to August were lower than from October to December ($P<0.01$). Regarding total sperm count and ejaculate volume, values increased in midsummer and decreased against in late autumn (Fig.1).

Table 1
Effect (P-value) of month of ejaculate collection on semen characteristics determined in raw (0 h) and cold-stored (24 and 48 h) semen in 15 stallions.

Parameter	P-value		
	0 h	24 h	48 h
Sperm concentration	<0.001*	-	-
Ejaculate volume	<0.001*	-	-
Total sperm count	<0.001*	-	-
Total motility	<0.001*	0.020*	0.094
Progressive motility	<0.001*	0.317	0.018*
Rapid cells	<0.001*	0.001*	0.135
Plasma membrane and acrosome intact sperm	-	0.004*	<0.001*
Sperm with low Ca^{2+} level	-	<0.001*	<0.001*
Mean DFI	<0.001*	<0.001*	<0.001*
SD of DFI	<0.001*	<0.001*	<0.001*
% DFI	<0.001*	<0.001*	<0.001*

*significant effect (P<0.05)

An effect (P<0.05) of the month was also apparent on all motility characteristics determined in raw semen, on total motility (%) and on rapid cells (%) after 24 h as well as on progressive motility (%) after 48 h of cold storage (Table 1, Fig. 2). Regarding all motility parameters measured in fresh semen, the values were lower in July than in any other month of the year with exception of the progressive motility in March (P<0.05). After 24 h of cold storage the values for rapid cells (%) were higher in April compared to January (P<0.05). After 48 h of storage, progressive motility (%) was higher in January and October than in July (P<0.05). The month of semen collection had a clear effect (P<0.05) on plasma membrane and acrosome integrity (PMAI %) as well as on viable sperm with low intracellular Ca^{+2} level (%) both determined after 24 and 48 h of storage (Table 1, Fig. 3). After 24 h of cold storage PMAI % was higher in October than in January (P<0.05), and after 48 h values were higher in ejaculates collected in September compared to January and February as well as from April to July (P<0.05). Regarding viable sperm with low intracellular Ca^{+2} level (%) after storage for 24 and 48 h, higher values were measured in December, January, February, March and October compared to April, June and July (P<0.01).
All SCSA^{TM} parameters assessed in raw frozen-thawed as well as in semen stored for 24 and 48 h were influenced (P<0.05) by the month of semen collection (Table 1, Fig. 4).
Mean DFI values in raw frozen-thawed semen were lower from August to November than in June and July (P<0.001) when the highest values were recorded. Mean DFI was lower during winter (January and February) compared to spring and early summer (April to July) (P<0.05) and lower in December than from April to September (P<0.001). Seasonal variations of SD of DFI and % DFI in raw frozen-thawed semen showed a similar but a less pronounced pattern as the mean DFI. SD of DFI was lower in February and October than in May and July as well as in March than July, respectively (P<0.05). % DFI in April and September was lower compared to June and in October than in July, respectively (P<0.05). After 24 h of cold storage mean DFI was lower in September and October when compared to January, February, May, July and November (P<0.05). Maximum mean DFI values were measured in July, these differed from March to June and from August to December, respectively (P<0.001). Regarding SD of DFI after 24 h of cold storage, minimal values were found in August and December, these differed when compared to values from February to July and to November (P<0.05). % DFI after 24 h of cold storage was lower in December than in February to July (P<0.05). After both 24 and 48 h of storage % DFI was lower in late summer (August to September) and early winter (December to January) than in spring (February to May) (P<0.05). After 48 h cold storage mean DFI was lower in spring (March to May) and autumn (September to December) compared to February, June and July (P<0.05). SD of DFI after 24

and 48 h of cold storage in December were lower when compared to late summer and spring (February to June) (P>0.05).

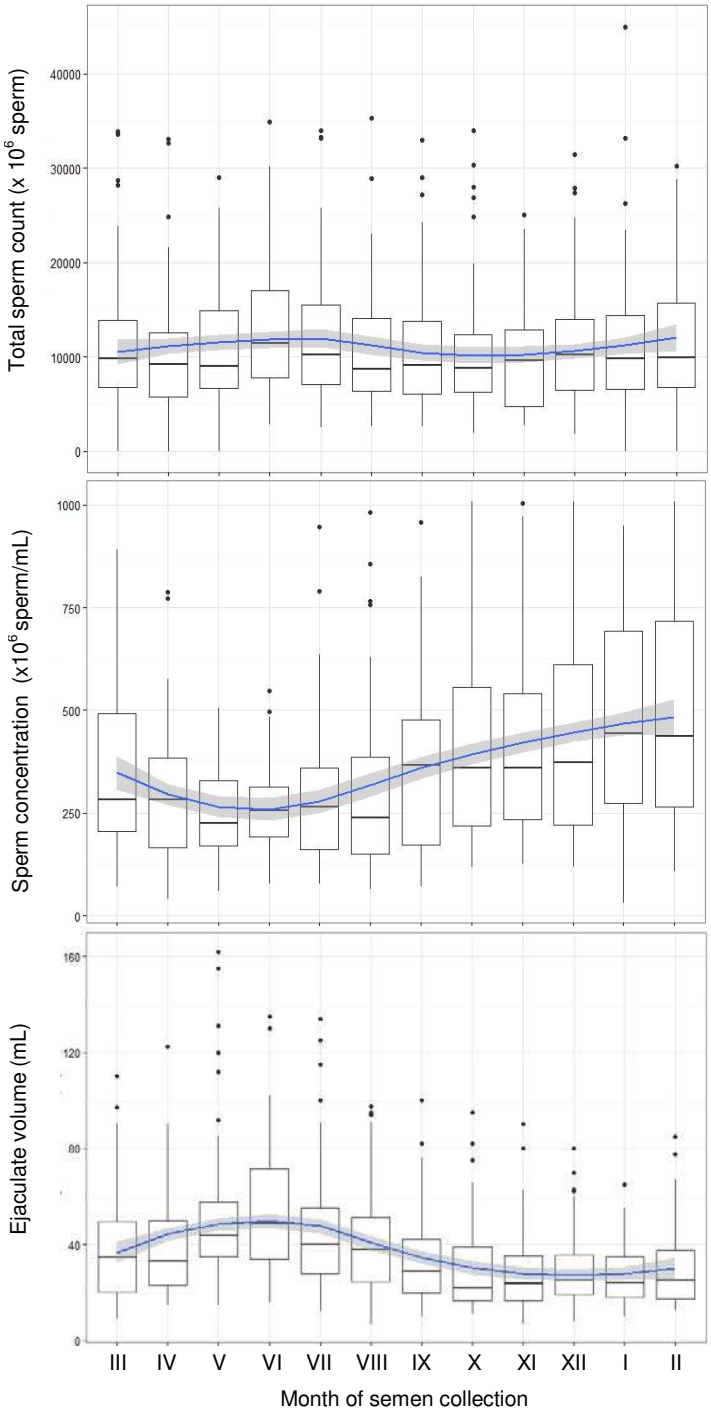

Fig.1. Box plots showing the annual variation of total sperm count, sperm concentration and volume of ejaculates collected weekly from 15 stallions.

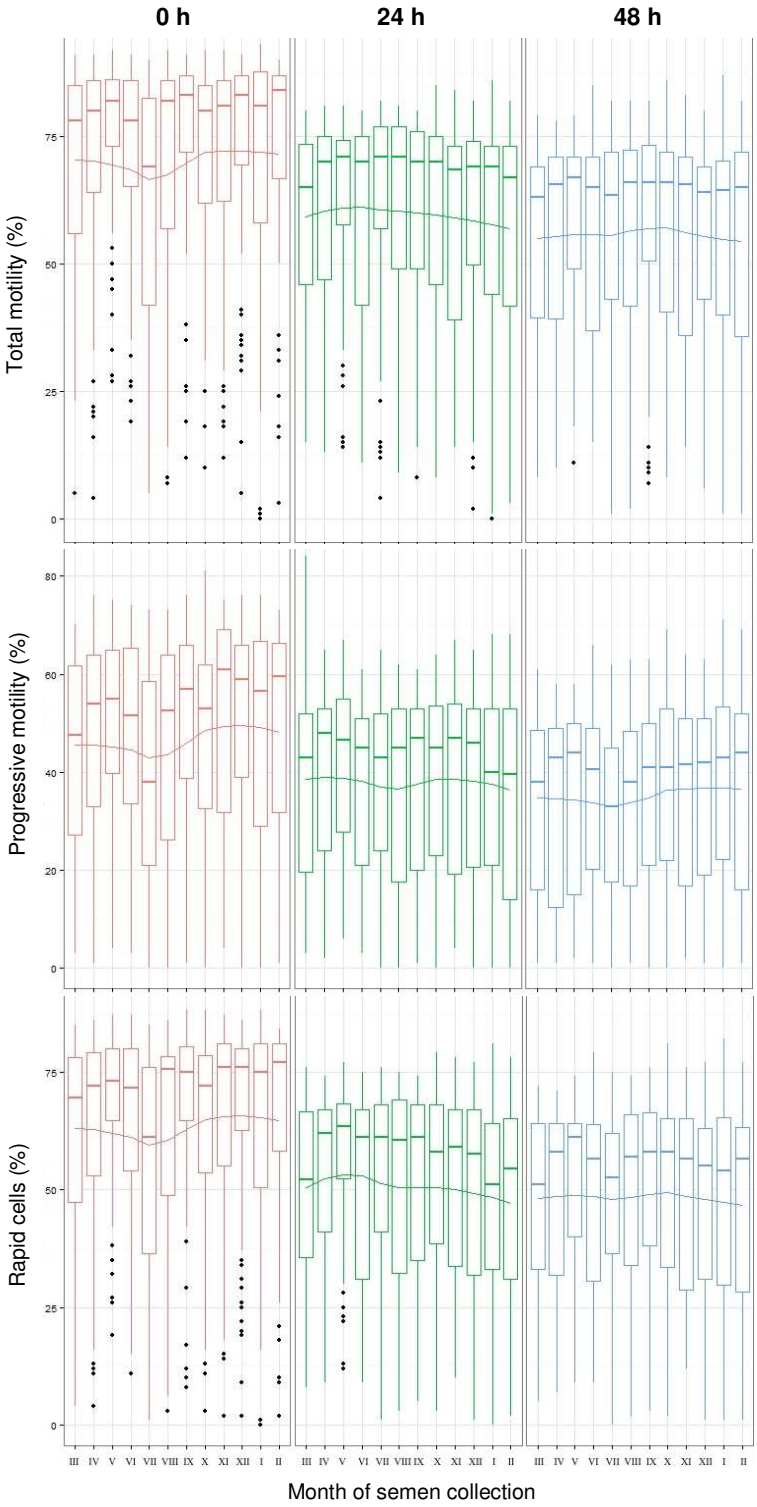

Fig.2. Box plots showing the annual variation of total motility (%), progressive motility (%) and rapid cells (%) in fresh (0 h) as well as in cold-stored semen (24 and 48 h) obtained weekly from 15 stallions.

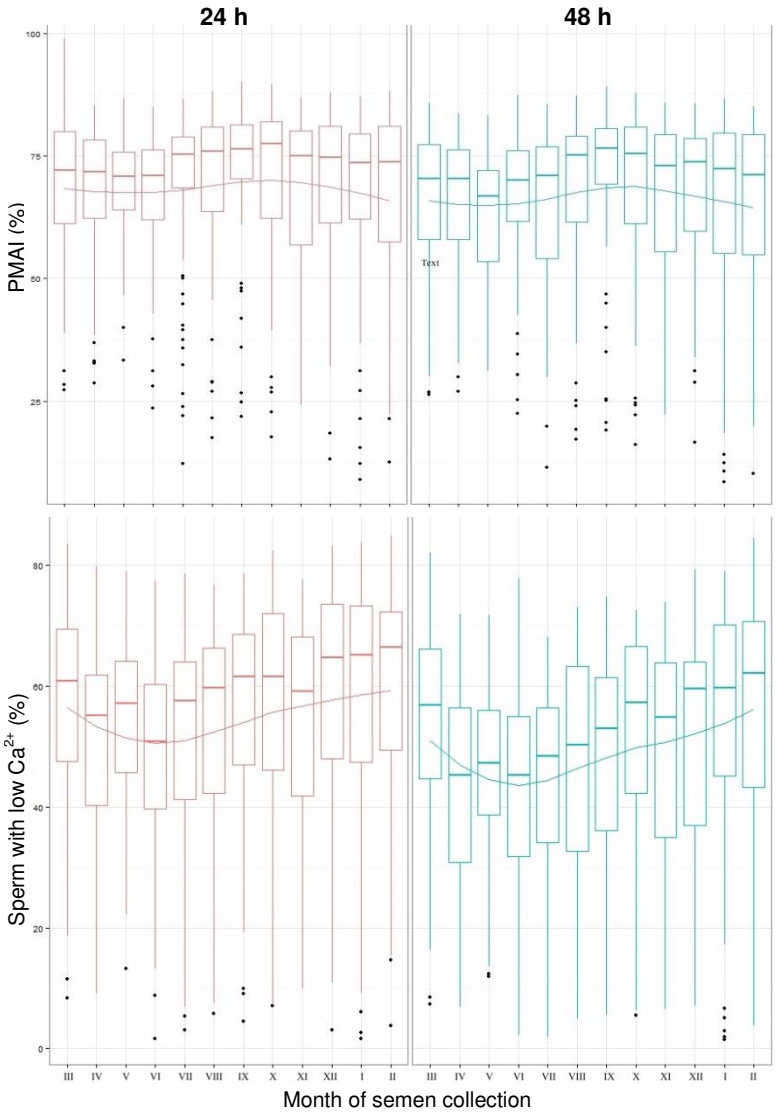

Fig. 3. Box plots showing the annual variation of plasma membrane and acrosome intact sperm (PMAI %) as well as of viable sperm with low Ca^{2+} level in cold-stored semen (24 and 48 h) obtained weekly from 15 stallions.

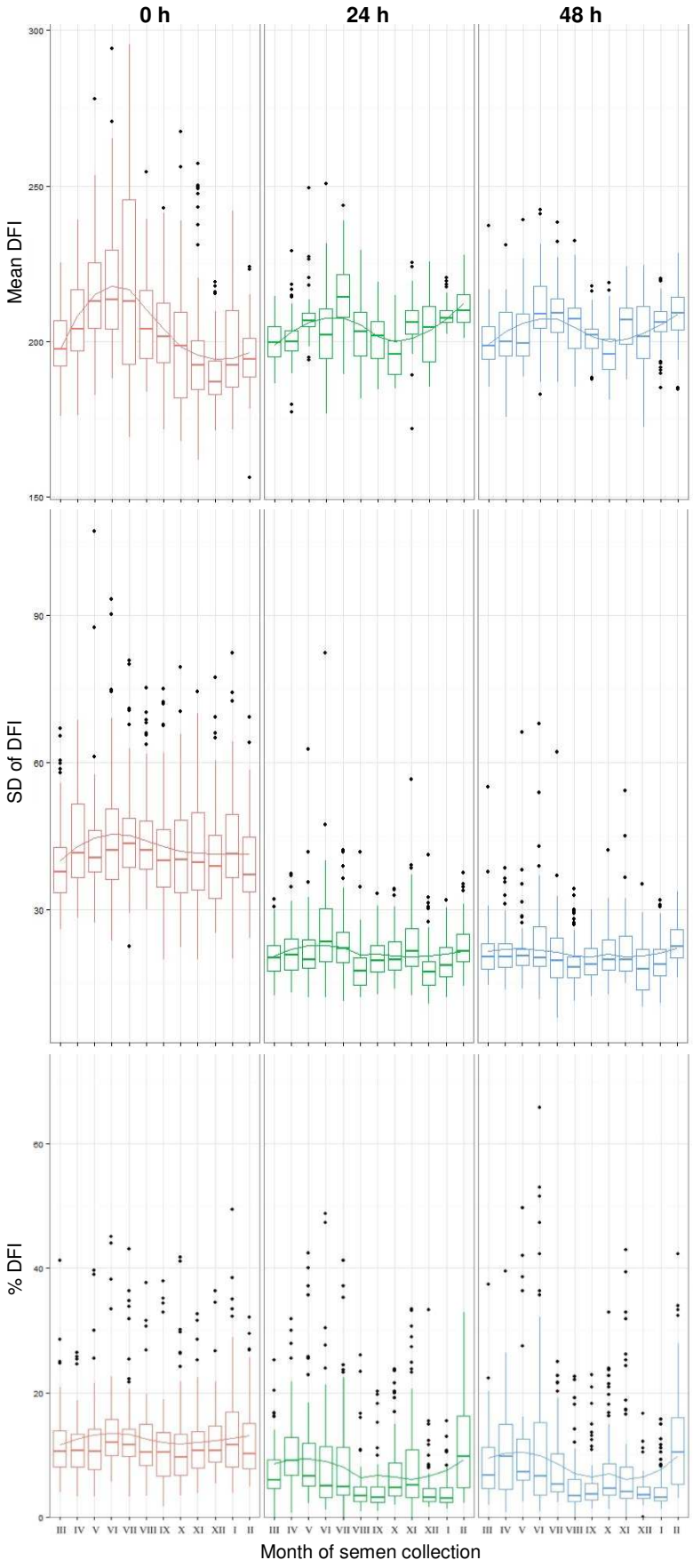

Fig. 4. Box plots showing the annual variation mean DFI, SD of DFI and % DFI of sperm in raw frozen-thawed (0 h) as well as in cold-stored semen (24 and 48 h) obtained weekly from 15 stallions.

3.3 Changes of semen characteristic during storage

Changes during storage of all evaluated sperm characteristics were affected by the interaction of storage time and month of semen collection ($P<0.001$). Regarding total motility (%) a significant ($P<0.05$) decrease was apparent between 0 and 24 h of storage in February and between 0 and 48 h in all months except July and August, respectively. A clear ($P<0.05$) drop of rapid cells between 0 and 24 h of storage was seen in January, February, September and December as well as between 0 and 48 h from January to April, in June and from September to December. Progressive motility (%) decreased only in November between 0 and 48 h storage. Between 24 and 48 h of cold storage no differences in motility parameters were apparent ($P>0.05$).

PMAI (%) decreased ($P<0.05$) between 24 and 48 h of storage in January, in March, from May to July and from October to December. Regarding viable sperm with low Ca^{2+} level (%), differences between 24 and 48 h of storage were significant ($P<0.05$) for all months. Significant ($P<0.05$) differences in mean DFI were apparent between 0 and 24 h in December, January, February, and June, and also between 0 and 48 h in December, January, February, May, June, and July. SD of DFI differed ($P<0.001$) between 0 and 24 h as well as between 0 and 24 h for all months. Higher ($P<0.05$) % DFI values were measured in raw-thawed semen (0 h) compared to semen cooled and stored for 24 and 48 h in January, March, June, August, September and December as well as in January, July, August, September and December, respectively. Between 24 and 48 h of cold storage no differences in SCSA^{TM} parameters were apparent ($P>0.05$).

3.4. Correlations between semen characteristics

In raw semen, a weak positive correlation ($0.3>r>0$, $P<0.05$) was found between all SCSA^{TM} parameters except for mean DFI and sperm concentration (weak negative correlation $-0.3<r<0$, $P<0.05$) and quantitative semen traits (volume, total sperm count). Moreover weak positive correlations were found between PMAI (%) and mean DFI at 48 h ($r=0.282$, $P<0.001$) as well as between PMAI (%) and SD of DFI at 48 h ($r=0.123$, $P<0.001$). Weak negative correlations were calculated between all SCSA^{TM} parameters and viable sperm with LOW-Ca^{+2} levels (%) at 24 and 48 h as well as PMAI (%) at 24 h and between % DFI and PMAI (%) at 48 h.

3.5. Serum testosterone concentrations

Serum testosterone concentration showed a clear seasonal pattern (Fig. 5) with high concentrations in spring and early summer (April to July) and low levels in late autumn and winter (October-February). A moderate correlation was evident between testosterone concentration and ejaculate volume ($r=0.408$, $P<0.001$). Weak correlations were found between testosterone concentration and total sperm count ($r=0.257$, $P<0.001$), mean DFI ($r=0.166$, $P<0.001$), sperm concentration ($r=-0.156$, $P<0.001$), progressive ($r=-0.121$, $P<0.001$) and total ($r=-0.113$, $P<0.003$) motility as well as well as rapid cells ($r=-0.119$, $P<0.002$) in raw semen.

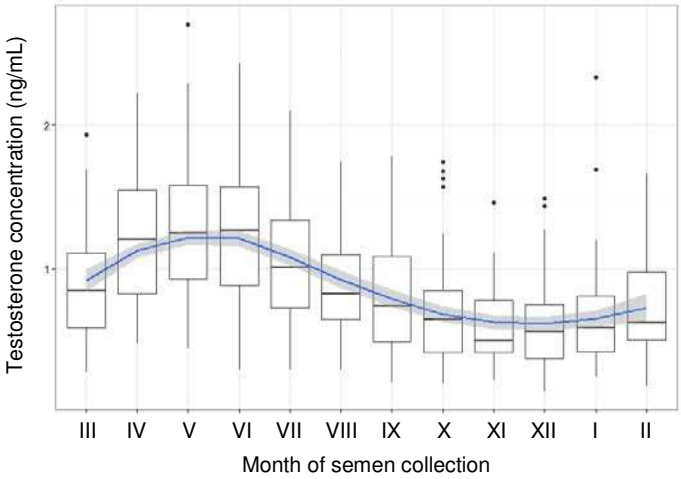

Fig. 5. Box plots showing the annual variation of the serum testosterone concentration determined weekly in 15 stallions.

3.6. Microclimatic conditions

Changes of microclimatic conditions observed during the experimental period were characteristic of Central European climate. Mean monthly temperature was highest in July (20.6 °C) and lowest in December (0.5 °C). A maximum air temperature of 33.9 °C has been measured on July-27 and values over 30 °C were recorded on 3 days in June, on 6 days in July and on 5 days in August. Monthly mean relative humidity fluctuated between 70.3 % (July) and 89.5 % (December). Highest and lowest daily sunshine hours were recorded in July (9.8 h) and in January (2.3 h), respectively.

4. Discussion

In this study, the annual variation of raw and cold-stored semen obtained weekly from 15 Franches-Montagnes stallions was investigated using computer-assisted sperm analysis and flow cytometry.

According to previous reports [1, 6, 8, 25], significant seasonal differences in routine raw semen traits were noted. In particular, ejaculate volume was higher and sperm concentration lower in summer compared to winter. This observation is probably the result of different testicular endocrine activity and secretion of the accessory glands which are stimulated by increasing day length [1, 3, 6-8]. Moreover sperm motility, a factor affecting stallion reproductive performance [6, 26-29], was lower in July than in any other month of the year. These findings support an earlier report using the same breed [1] but differ from other studies using pony [8], Standardbred [6] or Warmblood [25] stallions, reporting highest sperm concentrations in autumn [8, 25] or in August [6] and lowest sperm motility during winter [6, 8, 25]. The conflicting results of different studies indicate that beside ejaculatory frequency also the breed may have an impact on seasonal changes of raw semen traits.

Regarding sperm motility in cold-stored semen, seasonal changes were not as pronounced as in raw semen. Semen processed in spring and stored for 24 h was improved compared to winter and after 48 h of storage progressive motility (%) was lowest in July. These findings are inconsistent with recently published reports showing a reduced longevity of cold-stored semen that was produced in February compared to November [9] or spring and summer [10], respectively. However, the short experimental period in these two studies (3 and 7 months, respectively) was probably insufficient to investigate seasonal effects.

In cold-stored semen plasma membrane and acrosome integrity (PMAI %) as well as the intracellular Ca^{2+} level of viable sperm were investigated using flow cytometry. Sperm viability has been shown to be related to the fertility of cool-shipped [13] and frozen-thawed [30] stallion semen. In our study highest values of PMAI (%) in stored semen were measured in early autumn when ejaculate volume was decreasing. Large and small amounts as well as

composition of seminal plasma may impair plasma membrane integrity and motility of cold-stored [16, 31, 32] as well as of cryopreserved semen [14, 25]. In addition to the viability also the Ca^{2+} content was determined in cold-stored sperm. Alterations of the plasma membrane during semen processing and storage induce premature capacitation accompanied by an increase in intracellular Ca^{2+} level. In bulls, the intracellular Ca^{2+} level evaluated in frozen-thawed semen has been shown to be negatively correlated with fertility [33]. Determination of viable sperm with low Ca^{2+} level in our study showed that values were improved in October and during winter compared to summer. As seminal plasma proteins are known to modulate capacitation [34], seasonal changes in composition and the large amount of seminal plasma present in the ejaculates collected in late spring and summer may therefore explain the increased intracellular Ca^{2+} level of viable sperm during this period.

DNA fragmentation of sperm was assessed by SCSA^{TM}, a test proven to be related to stallion fertility [12, 13]. In general, susceptibility to DNA denaturation was higher in late spring and summer compared to autumn and winter when ambient temperature was lower. These findings are contrary to other studies reporting no seasonal variation [15, 35] or a slightly enhanced susceptibility of sperm DNA to denaturation in December [14]. Possible explanations for the conflicting results are that the different investigations were performed only during short periods in the breeding and non-breeding season and using small numbers of ejaculates and stallions [14, 35] as well as different breeds [14, 15, 35]. In the present study however, 15 stallions of the same breed were kept under identical conditions and ejaculates were evaluated weekly during a whole year enabling to detect seasonal changes. The increased susceptibility to DNA fragmentation observed during summer in our study could be attributed to the high ambient temperature exceeding 30 °C in June and July thus impairing scrotal thermoregulation. Scrotal heat stress can alter sperm chromatin structure in equine sperm and is associated with a decrease in protamine disulfide bonding [36]. SCSA^{TM} was performed in frozen raw semen immediately after thawing as well as in extended semen after 24 and 48 h of cold storage. Unexpectedly % DFI was often higher in raw frozen-thawed compared to cold-stored semen especially in ejaculates collected during winter and summer. This observation contradicts the generally valid assumption that sperm chromatin is not altered by the freeze/thaw procedure when evaluated by SCSA^{TM} [11]. Other than in mouse or human sperm however, our results and also a recent report using bull semen [37] show that the cryopreservation process may cause some degree of DNA damage in equine and in bovine sperm. From literature it is known that the cryopreservation process increases oxygen species (ROS) generation in the mitochondria [38, 39]. In equine sperm, unbalanced ROS leads to the production of toxic aldehyde adducts such as 4-hydroxynoneal (4-HNE) [39, 40] accelerating sperm senescence and resulting in a reduced functionality [39] and in promoting DNA fragmentation [37].

DNA fragmentation assessed after 24 and 48 h of cold storage was not influenced by storage time. This is in agreement with the result obtained by Love et al. 2002 [31] who found no changes in DNA denaturation in sperm of normal fertile stallions stored up to 46 h at 5 °C. Semen dilution and compounds present in the extender media may prevent sperm from DNA alterations induced by the seminal plasma [41].

Regarding quality changes during storage a clear drop of rapid cells was apparent between 0 and 24 h of cold storage during winter as well as between 0 and 48 h in autumn, winter and early spring, respectively. In the present study seminal plasma was not removed before semen storage. As it is known that this can impair the motility of chilled semen, especially when INRA96^{TM} is used as extender and semen is stored for more than 24 h [31], the effect of centrifugation on seasonal changes of the quality of cold-stored semen needs further investigation.

In conclusion, this study demonstrates a clear effect of season on sperm DNA fragmentation and on the characteristics of raw and cold-stored semen. A reduced quality of raw and cold-stored semen was recorded in midsummer when low sperm motility and viability were combined with an elevated DNA fragmentation and Ca^{2+} level of sperm.

Conflict of interest

None of the authors have any financial and personal relationships with other people or organizations that could inappropriately influence the study.

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